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## Identification of novel myeloma-specific XBP1 peptides able to generate cytotoxic T lymphocytes: A potential therapeutic application in multiple myeloma

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### Abstract

The purpose of these studies was to identify HLA-A2<sup>+</sup> immunogenic peptides derived from XBP1 antigens to induce a multiple myeloma (MM)-specific immune response. Six native peptides from non-spliced XBP1 antigen and three native peptides from spliced XBP1 antigen were selected and evaluated for their HLA-A2 specificity. Among them, XBP1<sub>184–192</sub>, XBP1 SP<sub>196–204</sub> and XBP1 SP<sub>367–375</sub> peptides showed the highest level of binding affinity, but not stability to HLA-A2 molecules. Novel heteroclitic XBP1 peptides, YISPWILAV or YLFPQLISV, demonstrated a significant improvement in HLA-A2 stability from their native XBP1<sub>184–192</sub> or XBP1 SP<sub>367–375</sub> peptide, respectively. Cytotoxic T lymphocytes generated by repeated stimulation of CD3<sup>+</sup> T cells with each HLA-A2-specific heteroclitic peptide showed an increased percentage of CD8<sup>+</sup> (cytotoxic) and CD69<sup>+</sup>/CD45RO<sup>+</sup> (activated memory) T cells and a lower percentage of CD4<sup>+</sup> (helper) and CD45RA<sup>+</sup>/CCR7<sup>+</sup> (naïve) T cells, which were distinct from the control T cells. Functionally, the CTLs demonstrated MM-specific and HLA-A2-restricted proliferation, IFN- $\gamma$  secretion and cytotoxic activity in response to MM cell lines and importantly, cytotoxicity against primary MM cells. These data demonstrate the distinct immunogenic characteristics of unique heteroclitic XBP1 peptides which induce MM-specific CTLs and highlights their potential application for immunotherapy to treat the patients with MM or its pre-malignant condition.

### INTRODUCTION

Multiple myeloma (MM) is a hematological malignancy characterized by the accumulation of clonogenic mature plasma cells in the bone marrow. Despite recent advances in treatment using new drugs developed in the past several years, the disease still remains incurable, thus novel therapeutic approaches are required to improve outcome.<sup>1–3</sup> Based on the success of allogeneic transplantation as well as graft-versus-myeloma responses following donor lymphocyte infusion, other immunotherapeutic approaches are being evaluated to treat the disease. The focus has been on augmenting and directing autologous anti-MM immune responses as allogeneic immune manipulations put patients at risk of developing graft-versus-host-disease with associated significant morbidity and mortality.<sup>4,5</sup> A number of

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### CONFLICT OF INTEREST

There are no competing financial conflicts of interest in relation to the work described.

targets have been investigated including use of patient-specific idiotype protein, MM cell lysates or MM cell-dendritic cell fusions.<sup>6–8</sup> However, these methods require generation of a patient-specific vaccine making its general applicability more difficult. Developing a peptide-based immunotherapy against specific MM-associated antigens would offer an attractive approach for a safe and effective immunotherapy. We report here on the identification and characterization of HLA-A2<sup>+</sup> peptides that elicit specific CTLs targeting XBP1 as the MM-specific antigen.

XBP1 is a transcription factor required for the terminal differentiation of B lymphocytes to plasma cells and is essential for immunoglobulin secretion.<sup>9, 10</sup> This antigen is a basic leucine zipper-containing transcription factor originally identified as a protein binding to the *cis*-acting X box region in the promoter of human MHC class II genes.<sup>11</sup> XBP1 mRNA is processed by IRE1, an endoplasmic reticulum (ER) transmembrane protein that contains endoribonuclease and cytoplasmic protein kinase domains in response to ER stress.<sup>12–14</sup> The mRNA spliced by IRE1 causes a reading frame shift which is translated into a spliced form of XBP1 protein that is an active transcription factor.<sup>15</sup> To date, XBP1 is the only transcription factor found to be essential for plasma cell differentiation. XBP1 is uniformly expressed in all MM cells and cell lines and is selectively induced by exposure to IL-6 and has been implicated in the proliferation of malignant plasma cells.<sup>16–19</sup> Microarray analyses have identified XBP1 as a differentially expressed gene between the plasma cells and monoclonal gammopathy of undetermined significance (MGUS) and MM cells.<sup>20</sup> Gene expression profiling studies have also confirmed the specific expression of XBP1 in MM.<sup>21</sup> A recent study shows that a splice variant of XBP1 plays a crucial role in normal plasma cell differentiation.<sup>22</sup> XBP1 splicing is recognized to occur in terminal B cell differentiation and correlates with plasma cell differentiation. In addition, there is evidence that spliced XBP1 supports restoration of immunoglobulin production in XBP1<sup>−/−</sup> B cells and induces IL-6 secretion in normal plasma cells development.<sup>23–25</sup> It has also been shown that the relative mRNA expression levels of spliced XBP1 compared to XBP1 are differentially expressed in myeloma compared with normal plasma cells.<sup>20</sup>

In this study, we propose the XBP1 transcription factor as a unique therapeutic target antigen for development of a MM-specific immunotherapy. We designed and characterized two heteroclitic peptides, YISPWILAV and YLFPQLISV, with improved HLA-A2 binding and stability from their respective native peptides, XBP1<sub>184–192</sub> (NISPWILAV) and XBP1<sub>SP367–375</sub> (ELFPQLISV). These heteroclitic peptides were able to induce XBP1 antigen-specific CTLs which specifically recognized, targeted, and lysed HLA-A2<sup>+</sup> MM cell lines. These data provides evidence of the potential of HLA-A2<sup>+</sup> XBP1 specific peptides for their clinical application as a peptide-based vaccine or in the production of XBP1-specific CTLs *ex vivo* for adoptive transfer therapy in patients with MM or its related pre-malignant diseases.

## MATERIALS AND METHODS

### Cell lines

The multiple myeloma lines, McCAR, MM1S and U266 were obtained from ATCC (Manassas, VA). The acute myeloid leukemia (AML) cell line, ML-2, was kindly provided by Dr. Y. Matsuo, Fujisaki Cell Center, Okayama, Japan. The T2 cell line, a human B and T cell hybrid expressing HLA-A2 molecules<sup>26</sup>, was provided by Dr. J. Mouldrem (University of Texas M. D. Anderson Cancer Center, Houston, TX) and was used as antigen presenting cells (APC). All cell lines were cultured in RPMI-1640 medium (Gibco-Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (FCS; BioWhittaker, Walkersville, MD), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco-Life Technologies).

## Reagents

Mouse anti-human CD3, CD4, CD8, CD14, CD40, CD45RA, CD45RO, CD69, CD86, CD107a, CCR7, HLA-A2 and HLA-DR monoclonal antibodies (mAbs) conjugated with FITC, PE, PerCP or APC were purchased from Becton Dickinson/Pharmingen (San Diego, CA). Mouse anti-human CD80 or CD83 mAbs conjugated with PE were purchased from Immunotech (Hialegha, FL). Recombinant human IL-2, IL-4, IFN- $\alpha$  and TNF- $\alpha$  were purchased from R&D Systems (Minneapolis, MN), and recombinant human GM-CSF was obtained from Immunex (Seattle, WA).

## Synthetic Peptides

Six native non-spliced XBP1 peptides including XBP1<sub>117–125</sub> (LLREKTHGL), XBP1<sub>184–192</sub> (NISPWILAV), XBP1<sub>189–197</sub> (ILAVLTLQI), XBP1<sub>192–200</sub> (VLTLQIQSL), XBP1<sub>110–118</sub> (KLLLENQLL), XBP1<sub>93–101</sub> (RMSELEQQV), three native spliced XBP1 peptides including XBP1 SP<sub>196–204</sub> (GILDNLDPV), XBP1 SP<sub>193–201</sub> (ILLGILDNL), XBP1 SP<sub>367–375</sub> (ELFPQLISV), and three heteroclitic peptides including XBP1<sub>184–192</sub> (YISPWILAV), XBP1 SP<sub>196–204</sub> (YILDNLDPV) and XBP1 SP<sub>367–375</sub> (YLFPQLISV) were examined as potential HLA-A2-specific peptides. Influenza virus matrix protein<sub>58–66</sub> (GILGFVFTL) was used as a control HLA-A2-specific peptide. All peptides were synthesized by standard fmoc (9-fluorenylmethyl-oxycarbonyl) chemistry, purified to >90% using reverse-phase chromatography and validated by mass-spectrometry for molecular weight (Biosynthesis, Lewisville, TX). Lyophilized peptides were dissolved in DMSO (Sigma, St. Louis, MO), diluted in AIM-V medium (Gibco-Life Technologies) and stored at  $-140^{\circ}\text{C}$ .

## Peptide Binding Assay

XBP1 peptides were evaluated for HLA-A2-specific binding with the T2 cell line. In the assay, T2 cells were washed, resuspended in serum-free AIM-V medium to a final concentration of  $1 \times 10^6$  cells/ml and transferred into wells of a 24-well tissue culture plate. The cells were pulsed with 50  $\mu\text{g/ml}$  of respective XBP1 peptide or 30  $\mu\text{g/ml}$  of influenza virus matrix protein<sub>58–66</sub> (GILGFVFTL) peptide plus 3  $\mu\text{g/ml}$  human  $\beta 2$ -microglobulin (Sigma) and incubated at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  in humidified air. Following overnight incubation, the cells were washed, stained with mouse anti-human HLA-A2-FITC mAb for 15 minutes at  $4^{\circ}\text{C}$ , washed and analyzed using a FACSsort<sup>TM</sup> flow cytometer with CellQuest<sup>TM</sup> v2.1 software (Becton Dickinson, San Jose, CA). Peptide binding to HLA-A2 was determined by the up-regulation of HLA-A2 molecules on T2 cells caused by HLA-A2 specific peptide binding and demonstrated by measuring mean fluorescence intensity (MFI) by flow cytometric analyses.

## Peptide Stability Assay

XBP1 peptides were examined for HLA-A2 binding stability using the T2 cell line. T2 cells were pulsed with the respective XBP1 peptide as described above. After overnight incubation, the cells were washed to remove unbound peptide and incubated with 10  $\mu\text{g/ml}$  Brefeldin A (Sigma) at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  for 1 hour to block cell surface expression of newly synthesized HLA-A2 molecules. Peptide/HLA-A2 binding stability was evaluated at 0, 2, 4, 6 and 18 hours post-Brefeldin A treatment. Following the incubation period, the cells were harvested, washed, stained with mouse anti-human HLA-A2-FITC mAb and analyzed by flow cytometry as described above.

## Generation of Monocyte-derived Dendritic Cells

Peripheral blood mononuclear cells (PBMC) were isolated by standard density gradient centrifugation over Ficoll-Paque<sup>TM</sup> Plus (Amersham Pharmacia Biotech AB, Uppsala

Sweden) from leukopaks obtained from HLA-A2<sup>+</sup> normal donors. Dendritic cells (DC) were generated from monocytes obtained as the adherent cell fraction. To generate DC, the monocytes were cultured for 7 days in the presence of 1,000 U/ml GM-CSF and 1,000 U/ml IL-4 in RPMI-1640 medium (Gibco-Life Technologies) supplemented with 10% FCS. Fresh media plus GM-CSF and IL-4 was added to the cultures every other day. Mature DC (mDC) were obtained by adding 1,000 U/ml IFN- $\alpha$  plus 10 ng/ml TNF- $\alpha$  along with fresh GM-CSF and IL-4 in 10% FCS-RPMI on day 7 and incubating additional three days. The mDC were harvested and used as antigen-presenting cells (APC) to generate XBP1 peptide specific-CTLs.

### Isolation of CD3<sup>+</sup> T cells

CD3<sup>+</sup> T cells were obtained from the non-adherent cell fraction (post-monocytes adherence) using the Pan T cell isolation kit from Miltenyi Biotec (Auburn, CA). In brief, T cell enrichment was accomplished by depletion of B cells, NK cells, early erythroid cells, platelets and basophils by labeling with a cocktail of hapten-conjugated CD11b, CD16, CD19, CD36 and CD56 antibodies and MACs microbeads coupled to an anti-hapten monoclonal antibody. The effluent (negative cell fraction) was collected from the column as enriched CD3<sup>+</sup> T cells. The purity of the enriched CD3<sup>+</sup> T cells was examined by flow cytometry and was found to be 94 $\pm$ 2% (mean  $\pm$  standard deviation).

### Induction of Peptide-specific CTLs

Peptide-specific CTLs were generated *ex vivo* by repeated stimulation of CD3<sup>+</sup> T lymphocytes obtained from HLA-A2<sup>+</sup> normal donors with XBP1 peptide-pulsed APC, either mDC or T2 cells. In brief, APC were washed and resuspended in serum-free medium AIM-V medium and pulsed overnight at 37°C and 5% CO<sub>2</sub> in humidified air with 50  $\mu$ g/ml of the appropriate XBP1 peptide. Peptide-pulsed APC were washed, counted, irradiated at 10 Gy and used to prime CD3<sup>+</sup> T cells at a 1:20 APC/peptide (stimulator)-to-CD3<sup>+</sup> T cell (responder) ratio in AIM-V medium supplemented with 10% human AB serum (BioWhittaker). The T cell cultures were restimulated every seven days with irradiated APC/peptide for a total of 4 cycles to generate XBP1 peptide-specific CTL (XBP1-CTL). IL-2 (50 U/ml) was added to the cultures two days after the second stimulation. Control CD3<sup>+</sup> T cell cultures (unstimulated) were maintained in AIM-V medium supplemented with 10% human AB serum containing 50 U/ml IL-2.

### Phenotypic analysis of the XBP1-CTLs or target/stimulatory cell lines

XBP1-CTLs were stained with CD4-PE or CD8-FITC mAbs for their phenotype analyses. In addition, they were stained with CD69-FITC/CD45RO-PE or CD45RA-FITC/CCR7-PE mouse mAbs. Alternatively, the multiple myeloma cell lines (McCAR, U266 and MM1S) along with an acute myeloid leukemia cell line (ML-2) were stained with HLA-A2-FITC mAb prior to use as target or stimulatory cells. CTLs or cell lines were stained with the appropriate mAbs, washed and analyzed by flow cytometry.

### IFN- $\gamma$ ELISA

IFN- $\gamma$  secretion by XBP1-CTLs was measured using a human IFN- $\gamma$  ELISA kit (BD Biosciences, San Diego, CA). For the assay, XBP1-CTLs were incubated with the MM (McCAR, MM1S) or AML (ML-2) cell lines for 24 hours at 37°C, 5% CO<sub>2</sub> in humidified air. The supernatants were harvested and stored at -20°C until analyzed by ELISA for IFN- $\gamma$  release by the XBP1-CTLs in response to the MM or AML cell lines used as stimulator cells. Briefly, purified IFN- $\gamma$  as standards or supernatants were transferred into wells of a 96-well plate pre-coated with a monoclonal anti-human IFN- $\gamma$  capture antibody and incubated for 2 hours at room temperature. After washing, the buffer containing detection

antibody and avidin-horseradish peroxidase conjugate was added to each well and incubated for 1 hour at room temperature. The wells were washed and developed by incubation with TMB substrate solution for 30 minutes. Stop solution was added to each well and the absorbance was determined at 450 nm with a VICTOR<sup>2</sup>-1420 multilabel counter (PerkinElmer, Wellesley, MA). The amount of IFN- $\gamma$  (pg/ml) present in the CTLs culture supernatant was calculated based on the standard curve

### Cell proliferation by Carboxy Fluorescein Succinimidyl Ester (CFSE) tracking

XBP1-CTLs were harvested, washed twice in PBS (Gibco-Life Technologies) and resuspended at  $1 \times 10^6$  cells/ml. CFSE (Molecular Probes, Eugene, OR), 5 mM stock solution in dimethyl sulfoxide, was added to the CTLs to give a final concentration of 5  $\mu$ M and incubated for 10 minutes at 37°C, 5% CO<sub>2</sub> protected from light. After incubation, 5 volumes of ice-cold PBS with 2% FCS (PBS-FCS) were added to the cells to quench the reaction. The cells were incubated for 5 minutes on ice, washed 3 $\times$  with PBS-FCS and resuspended in fresh AIM-V media supplemented with 10% human AB serum. The CFSE labeled XBP1-CTLs ( $2 \times 10^6$  cells/ml) were co-incubated with McCAR, MM1S or ML-2 cells ( $2 \times 10^5$  cells/ml) or media alone at 37°C and 5% CO<sub>2</sub> in humidified air. The CFSE-labeled CTLs were harvested and examined on day 4 to determine their proliferation in response to the antigen present on the tumor cell lines. CTLs that proliferate in response to antigen show a reduction in CFSE fluorescence intensity, which was measured directly by flow cytometry.

### Cytotoxicity Assay

The cytotoxic activity of the XBP1-CTLs was measured using a calcein-release assay as described elsewhere.<sup>27</sup> Briefly,  $3 \times 10^5$  target cells (McCAR, U266, MM1S, ML-2 or primary CD138<sup>+</sup> cells from HLA-A2<sup>+</sup> MM patients) were incubated with 10 mM calcein-AM (Molecular Probes) for 30 minutes at 37°C, washed 3 $\times$  and resuspended in PBS containing 5% FCS. The calcein-AM labeled target cells ( $5 \times 10^3$ /well) were incubated with the XBP1-CTLs at various effector:target cell ratios in 96-well U-bottom microtiter plates (triplicate wells/sample). Plates were incubated for 4 hours at 37°C, 5% CO<sub>2</sub>. After incubation, the cells were pelleted by centrifugation at 1,000 rpm for 5 minutes and 100  $\mu$ l of the supernatant were transferred from each well to 96-well flat-bottomed plates. The fluorescence of each supernatant was monitored at 490 nm excitation and 520 nm emission wavelength using a VICTOR<sup>2</sup>-1420 multilabel counter (Perkin-Elmer). Maximum release was obtained from detergent-released target cells and spontaneous release from target cells incubated in the absence of effector cells. The cytotoxicity of the XBP1-CTLs was calculated as follows: % Specific Lysis = [(experimental release – spontaneous release)  $\div$  maximum release – spontaneous release].

### CD107 assay

The CD107 assay was setup with minor modifications to evaluate the functional activity of the XBP1-CTLs.<sup>28</sup> Primary CD138<sup>+</sup> MM cells from bone marrow mononuclear cells were isolated from HLA-A2<sup>+</sup> myeloma patients using RoboSep<sup>®</sup> CD138 positive immunomagnetic selection technology per manufacturer's instructions (STEMCELL Technologies, Vancouver, BC, Canada). The XBP-1 CTLs were co-incubated with HLA-A2<sup>+</sup>/CD138<sup>+</sup> MM cells at a ratio of 1 CTL:5 CD138<sup>+</sup> MM cells in a 96 well round bottom plate. At the same time, mouse anti-human CD107a-FITC mAb was added to each well. After 1 hour incubation, Brefeldin A (BD) and Monensin (BD) were added to the cells and cells were incubated for an additional 4 hours. Following incubation, the cells were harvested, washed, stained with mouse anti-human CD8-PE mAb for 30 minutes at 4°C, washed and analyzed by flow cytometry. The expression of CD107a by CD8<sup>+</sup> T cells was determined as a measure of degranulation of XBP1-CTLs in response to primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> MM cells.



## Statistical Analysis

Results are presented as mean  $\pm$  SE. Groups were compared using unpaired Student's t-test. Differences were considered significant when  $p < 0.05$ .

## RESULTS

### Non-spliced and spliced XBP1 peptides display high level of binding and stability to HLA-A2 molecules

The full length amino acid sequences from non-spliced and spliced XBP1 proteins were analyzed using the search software SYFPEITHI to predict HLA-A2-binding peptides, followed by the BIMAS and NetCTL programs to select peptides with extended half-time disassociation rates, proteasomal C terminal cleavage and TAP transport. A total of six native peptides from non-spliced XBP1 protein [XBP1<sub>117–125</sub> (LLREKTHGL), XBP1<sub>184–192</sub> (NISPWILAV), XBP1<sub>189–197</sub> (ILAVLTLQI), XBP1<sub>192–200</sub> (VLTLLQIQL), XBP1<sub>110–118</sub> (KLLLENQLL), XBP1<sub>93–101</sub> (RMSELEQQV)] and three native peptides [XBP1 SP<sub>196–204</sub> (GILDNLDPV), XBP1 SP<sub>193–201</sub> (ILLGILDNL), XBP1 SP<sub>367–375</sub> (ELFPQLISV)] from spliced XBP1 protein were selected for evaluation of HLA-A2-specific binding affinity (Table 1 and Table 2). The T2 peptide-binding assay was used to assess the HLA-A2 affinity of the XBP1 peptides.

Among the XBP1 peptides derived from non-spliced XBP1 protein, XBP1<sub>184–192</sub> (NISPWILAV) showed the highest HLA-A2 binding (MFI =  $720 \pm 140$ ) which was greater than the influenza virus matrix protein<sub>58–66</sub> peptide (GILGFVFTL) (MFI =  $604 \pm 10$ ) used as a HLA-A2-specific positive control (Figure 1a). However, initial peptide stability studies revealed that the HLA-A2 binding of the native XBP1<sub>184–192</sub> (NISPWILAV) peptide was unstable compared to influenza virus matrix protein<sub>58–66</sub> peptide (GILGFVFTL) (data not shown). In order to improve the stability of the native XBP1<sub>184–192</sub> (NISPWILAV) peptide binding to HLA-A2 molecules, we designed the heteroclitic peptide (YISPWILAV) by substitution of the tyrosine (Y) for asparagine (N) in position 1 from the native peptide. The HLA-A2 binding stability of the heteroclitic XBP1<sub>184–192</sub> peptide was significantly improved ( $> 6$  hours) compared to the native XBP1<sub>184–192</sub> peptide (Figure 1b). In addition, the stability of the heteroclitic XBP1<sub>184–192</sub> peptide to HLA-A2 molecule was greater than the control influenza virus matrix protein<sub>58–66</sub> (GILGFVFTL), demonstrating that the peptide modification improved HLA-A2 stability for the heteroclitic XBP1<sub>184–192</sub> peptide.

Among the peptides derived from spliced XBP1 protein, XBP1 SP<sub>196–204</sub> (GILDNLDPV) and XBP1 SP<sub>367–375</sub> (ELFPQLISV) showed the highest levels of HLA-A2 binding affinity (MFI =  $762 \pm 167$ , MFI =  $785 \pm 84$ , respectively) which were comparable to the control influenza virus matrix protein<sub>58–66</sub> peptide (Figure 1c). The peptide sequences of XBP1 SP<sub>196–204</sub> and XBP1 SP<sub>367–375</sub> were modified to enhance their binding stability to HLA-A2 molecules. Improvement in HLA-A2 binding stability was observed with the heteroclitic peptide, YLFPQLISV, which was modified from the native XBP1 SP<sub>367–375</sub> (ELFPQLISV) (Figure 1d). The HLA-A2 binding stability was not improved for the other heteroclitic peptide, YILDNLDPV, which was derived from the native XBP1 SP<sub>196–204</sub> (GILDNLDPV) peptide (data not shown). Therefore, the heteroclitic XBP1<sub>184–192</sub> (YISPWILAV) and heteroclitic XBP1 SP<sub>367–375</sub> (YLFPQLISV) peptides were selected for further evaluation of their immunogenic potential to generate MM-specific CTLs.

### The XBP1-specific CTLs display a distinct phenotype from unstimulated T cells

Antigen-specific CTLs can be phenotypically identified as activated/memory T cells from naïve T cells by their expression of distinct cell surface antigens. We analyzed the percentage of activated/memory (CD69<sup>+</sup>/CD45RO<sup>+</sup>) and naïve (CD45RA<sup>+</sup>/CCR7<sup>+</sup>) T cells

along with the T cell subsets (CD4<sup>+</sup>, CD8<sup>+</sup>) in the CD3<sup>+</sup> T cell cultures stimulated with either heteroclitic XBP1<sub>184–192</sub> (YISPWILAV) or heteroclitic XBP1 SP<sub>367–375</sub> (YLFPQLISV) peptide. The CD3<sup>+</sup> T cells stimulated with respective heteroclitic XBP1 peptide induced a significantly higher percentage of CD8<sup>+</sup> T cells (heteroclitic XBP1<sub>184–192</sub> stimulated - 81%; heteroclitic XBP1 SP<sub>367–375</sub> stimulated - 75%) compared to control T cells (no stimulation - 33%). We also observed a lower percentage of CD4<sup>+</sup> T cells (heteroclitic XBP1<sub>184–192</sub> stimulated - 14%; heteroclitic XBP1 SP<sub>367–375</sub> stimulated - 18%) in the XBP1-CTL cultures as compared to the control CD3<sup>+</sup> T cell cultures (no peptide stimulation = 64%) (Figure 2). The XBP1-CTLs or control T cells were further examined for naïve (CD45RA<sup>+</sup>/CCR7<sup>+</sup>) or activated memory (CD69<sup>+</sup>/CD45RO<sup>+</sup>) cell subtypes. The cell population expressing an activated memory (CD69<sup>+</sup>/CD45RO<sup>+</sup>) phenotype was significantly higher in the XBP1-CTLs (heteroclitic XBP1<sub>184–192</sub> stimulated - 62%; heteroclitic XBP1 SP<sub>367–375</sub> stimulated - 64%) compared to control T cells (no stimulation - 4%) obtained from the same donor (Figure 2). In addition, the control T cell cultures showed 24% naïve (CD45RA<sup>+</sup>/CCR7<sup>+</sup>) cells, whereas only 2% of the XBP1-CTLs showed this subset (data not shown). Therefore, these results demonstrate that stimulation with heteroclitic XBP1 peptides lead to an expansion of the CD8<sup>+</sup> T cell subset with an activated/memory (CD69<sup>+</sup>/CD45RO<sup>+</sup>) phenotype characteristic of effector cells.

### **XBP1-CTLs produce IFN- $\gamma$ by stimulation with HLA-A2<sup>+</sup> myeloma cells**

To characterize the anti-tumor response, antigen-specificity and HLA-A2-restriction of the heteroclitic XBP1 peptide induced CTLs, we evaluated their ability to produce IFN- $\gamma$  following stimulation with various tumor cell lines including McCAR (MM; HLA-A2<sup>+</sup>/XBP1<sup>+</sup>), MM1S (MM; HLA-A2<sup>-</sup>/XBP1<sup>+</sup>) or ML-2 (AML; HLA-A2<sup>+</sup>/XBP1<sup>-</sup>). Supernatants collected after 24 hours incubation of XBP1-CTLs with the respective tumor cell line were evaluated for IFN- $\gamma$  (pg/ml) by ELISA. We observed a dramatic increase in IFN- $\gamma$  secretion by both heteroclitic XBP1-CTLs stimulated McCAR (HLA-A2<sup>+</sup>/XBP1<sup>+</sup>) myeloma cells compared to the CTLs alone without stimulation (Figure 3). Stimulation with the MHC mismatched MM1S (MM; HLA-A2<sup>-</sup>/XBP1<sup>+</sup>) or the antigen mismatched ML-2 (AML; HLA-A2<sup>+</sup>/XBP1<sup>-</sup>) cell lines did not induce IFN- $\gamma$  secretion by either of the heteroclitic XBP1-CTLs. These results offer evidence of an antigen-specific and HLA-A2-restricted IFN- $\gamma$  secretion by the CTLs generated with heteroclitic XBP1<sub>184–192</sub> (YISPWILAV) or heteroclitic XBP1 SP<sub>367–375</sub> (YLFPQLISV) peptides.

### **XBP1-CTLs show specific cell proliferation in response to HLA-A2<sup>+</sup> myeloma cells**

A CFSE-based assay was used to analyze the proliferation of heteroclitic XBP1-CTLs upon stimulation with various irradiated tumor cell lines. Cell proliferation was analyzed by measuring the decrease in fluorescence of the CFSE-labeled CTLs by flow cytometry (M1-gated cells). Antigen-specific and HLA-restricted cell proliferation was observed by the XBP1-CTLs in response to HLA-A2<sup>+</sup>/XBP1<sup>+</sup> McCAR (heteroclitic XBP1<sub>184–192</sub>-CTLs: 55%, heteroclitic XBP1 SP<sub>367–375</sub>-CTLs: 42%) compared to the control CTLs without stimulation (heteroclitic XBP1<sub>184–192</sub>-CTLs: 5%, heteroclitic XBP1 SP<sub>367–375</sub>-CTLs: 2%) (Figure 4). However, the XBP-1 CTLs did not proliferate in response to the MHC mismatched MM1S (MM; HLA-A2<sup>-</sup>/XBP1<sup>+</sup>) or the antigen mismatched ML-2 (AML; HLA-A2<sup>+</sup>/XBP1<sup>-</sup>) cell lines. Taken together, our data demonstrated the specific cell proliferation of XBP1-CTLs in response to myeloma cells in antigen-specific and HLA-A2-restricted manner.

### **XBP1-CTLs induce specific lysis of HLA-A2<sup>+</sup>/XBP1<sup>+</sup> myeloma cell lines**

Next, we demonstrate the ability of the heteroclitic XBP1<sub>184–192</sub> or heteroclitic XBP1 SP<sub>367–375</sub> peptide-specific CTLs to specifically target and lyse MM cells using a calcein-



release cytotoxicity assay. Heteroclitic XBP1<sub>184-192</sub>-CTLs (Figure 5a) as well as heteroclitic XBP1 SP<sub>367-375</sub>-CTLs (Figure 5b) demonstrated effective lysis of the HLA-A2<sup>+</sup>/XBP1<sup>+</sup> McCAR cells at various effector: target cell ratios (9–50% specific lysis by heteroclitic XBP1<sub>184-192</sub>-CTLs; 15–69% by heteroclitic XBP1 SP<sub>367-375</sub>-CTLs). In addition, an alternative HLA-A2<sup>+</sup>/XBP1<sup>+</sup> myeloma cell line, U266, was effectively lysed by the heteroclitic XBP1<sub>184-192</sub>-CTLs (16–74% specific lysis) and heteroclitic XBP1 SP<sub>367-375</sub>-CTLs (18–83% specific lysis). Interestingly, the cytotoxic activity against McCAR or U266 myeloma cell lines was greater for the CTLs induced with the XBP1 SP<sub>367-375</sub> peptide derived from spliced XBP1 protein as compared to those induced with the XBP1<sub>184-192</sub> peptide from non-spliced protein. The heteroclitic XBP1<sub>184-192</sub>-CTLs or heteroclitic XBP1 SP<sub>367-375</sub>-CTLs did not lyse the antigen mismatched ML-2 (AML; HLA-A2<sup>+</sup>/XBP1<sup>+</sup>) or MHC mismatched MM1S (MM; HLA-A2<sup>+</sup>/XBP1<sup>+</sup>) cell lines, demonstrating that the cytotoxic activity of both XBP1-CTLs is antigen-specific and HLA-restricted. The XBP1-CTLs did not lyse the NK-sensitive K562 cell line (data not shown), suggesting that the cytotoxicity of XBP1-CTLs was not due to an activated NK cell population.

### **XBP1-CTLs induce degranulation and cytotoxicity against primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> MM cells**

To evaluate effectiveness of XBP1-CTLs against primary patient MM cells, we evaluated CTL degranulation in response to target cell recognition by measuring expression of CD107a, a surface antigen transiently present on the cell surface after release of cytotoxic granules. XBP1-CTLs incubated with primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> myeloma cells in the presence of CD107a-FITC mAb were stained with CD8-PE mAb and evaluated by flow cytometry. Both heteroclitic XBP1<sub>184-192</sub>-CTLs (Figure 6a) and heteroclitic XBP1 SP<sub>367-375</sub>-CTLs (Figure 6b) displayed increased CD107a expression on CD8<sup>+</sup> T cells in response to interaction with primary CD138<sup>+</sup> MM cells isolated from three HLA-A2<sup>+</sup> MM patients. CD107a expression on CD8<sup>+</sup> T cells from heteroclitic XBP1<sub>184-192</sub> CTLs ranged between 4 – 19% while heteroclitic XBP1 SP<sub>367-375</sub> CTLs ranged between 7 – 16%. In contrast, unstimulated T cells from the same donor showed minimal expression of CD107a on CD8<sup>+</sup> cells in response to stimulation with the primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> cells from MM patients (0 – 1%).

Importantly, XBP1-CTLs demonstrated direct cytotoxicity against primary CD138<sup>+</sup> MM cells isolated from HLA-A2<sup>+</sup> patients using a calcein-release cytotoxicity assay. We evaluated cytotoxicity of heteroclitic XBP1<sub>184-192</sub>-CTLs and heteroclitic XBP1 SP<sub>367-375</sub>-CTLs generated from two different donors against primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> MM cells from two patients (Figures 7a and 7b). XBP1-CTLs generated from both donors displayed the highest cytotoxic activity against HLA-A2<sup>+</sup>/CD138<sup>+</sup> cells at an effector:target cell ratio of 60:1. The control unstimulated T cells did not lyse primary CD138<sup>+</sup> MM cells from the HLA-A2<sup>+</sup> MM patients. These data demonstrates the functional activity of the heteroclitic XBP1<sub>184-192</sub> and XBP1 SP<sub>367-375</sub>-CTLs against the primary MM cells through degranulation and subsequent cytolytic activity.

## **DISCUSSION**

XBP1 is a basic leucine zipper-containing transcription factor required for the terminal differentiation of B lymphocytes to plasma cells.<sup>9, 29–30</sup> It has been suggested that the high amount of immunoglobulin molecules produced in the plasma cells evokes ER stress, which in turn activates IRE1-mediated XBP1 expression and subsequently mRNA splicing during the plasma cell differentiation.<sup>12, 15–17</sup> The active XBP1 intracellular protein is thus highly expressed only in the plasma cells through both transcriptional and post-transcriptional mechanisms.<sup>14, 22, 31</sup> Additionally, XBP1 expression is higher in MM, compared to MGUS.<sup>30, 32</sup> This unique profile of XBP1 over-expression provides an opportunity to

develop an antigen-specific immunotherapy for the patients with MM or the pre-malignant diseases such as MGUS and smoldering multiple myeloma.

Here, we report on the identification and characterization of novel heteroclitic HLA-A2-specific XBP1 epitopes that induce specific anti-tumor CTLs that specifically target MM cells. The peptide identification was performed by screening the full-length sequences of non-spliced (260 amino acids) or spliced (375 amino acids) XBP1 protein to predict HLA-A2-specific peptides. We further screened the HLA-A2 anchor residues of the predicted peptides to avoid the epitopes containing amino acid which causes negative effects on HLA-A2 binding.<sup>33</sup> Based on the examination of HLA-A2 anchor residues, optimal peptides were selected from spliced or non-spliced XBP1 protein and evaluated for their HLA-A2 affinity. Among them, non-spliced XBP1<sub>184–192</sub> (NISPWILAV) and spliced XBP1<sub>367–375</sub> (ELFPQLISV) peptides demonstrated a high level of binding to HLA-A2 molecules.

Previously, investigators have shown that increased peptide stability to MHC molecules enhances its immunogenicity.<sup>34–38</sup> In these studies, we identified heteroclitic XBP1-specific peptides (YISPWILAV, YLFPQLISV) by altering the primary anchor residue of the native non-spliced XBP1<sub>184–192</sub> (NISPWILAV) and spliced XBP1<sub>367–375</sub> (ELFPQLISV) peptides, respectively. These heteroclitic XBP1 peptides demonstrated improved HLA-A2 binding stability on antigen-presenting cells while maintaining their recognition by T-cell receptors and ability to activate the antigen-specific T cells. In addition, the sequence of the heteroclitic analogues were confirmed not to overlap with sequences of peptides derived from other normal proteins, so that the risk of targeting the normal proteins is avoided by the heteroclitic XBP1 peptide-specific CTLs.

The challenge in targeting XBP1 antigen is to break T-cell tolerance to this plasma cell-specific antigen. Lotz et al.<sup>39</sup> suggested that the HLA-A2-restricted XBP1-specific human T cell repertoire is affected by partial self-tolerance. We examined whether the heteroclitic XBP1 peptides are able to break T-cell tolerance to this plasma cell-specific self-antigen. Our particular additional interest was the ability of the CTLs induced by heteroclitic XBP1 peptide to recognize the native XBP1 peptide which potentially presented on MM cells. In order to address these issues, we generated CTLs specific to the respective heteroclitic XBP1 peptide and evaluated their immunogenicity against tumor cell lines. This was performed by repeated stimulation of HLA-A2<sup>+</sup> normal CD3<sup>+</sup> T lymphocytes with APC pulsed with either heteroclitic XBP1<sub>184–192</sub> (YISPWILAV) or heteroclitic XBP1 SP<sub>367–375</sub> (YLFPQLISV) peptide. The resulting cell cultures displayed an altered T cell phenotype including an increased proportion of CD8<sup>+</sup> and activated/memory (CD69<sup>+</sup>/CD45RO<sup>+</sup>) T cells and a decreased proportion of CD4<sup>+</sup> and naïve (CD45RA<sup>+</sup>/CCR7<sup>+</sup>) T cells (Figure 2). These observations confirm results by other investigators that *ex vivo* generated tumor associated antigen-specific CTLs have distinct phenotypes of effector cells.<sup>7, 40–42</sup> In functional analysis, we demonstrated antitumor responses of the XBP1-CTLs against myeloma cells in XBP1-specific and HLA-A2 restricted manner by their IFN- $\gamma$  secretion (Figure 3), cell proliferation (Figure 4) and cytotoxicity (Figure 5). Importantly, we demonstrate the functional activity of heteroclitic XBP1<sub>184–192</sub> and XBP1 SP<sub>367–375</sub> CTLs to both degranulate and lyse primary CD138<sup>+</sup> cells from HLA-A2<sup>+</sup> MM patients (Figures 6 and 7). Interestingly, the cytotoxic activity of the spliced heteroclitic SP<sub>367–375</sub> peptide-specific CTLs was higher than the non-spliced XBP1<sub>184–192</sub> peptide-specific CTLs against both MM cell lines (Figure 5) as well as primary MM cells (Figure 7). These results may be explained by the crucial role of spliced variant of XBP1 in plasma cell differentiation and its over-expression in malignant myeloma cells.<sup>24, 25</sup> Taken together, the altered T cell phenotype enriched for CD8<sup>+</sup> and activated/memory T cell subtypes support the MM-specific functional activities of the CTLs generated by the heteroclitic XBP1 peptides.

An immunotherapeutic approach using the immunogenic heteroclitic XBP1 peptides can be translated into several different clinical applications including peptide vaccinations or adoptive transfer of *ex vivo* generated peptide-specific CTLs.<sup>43–47</sup> Infusion of peptide-specific CTLs in MM patients could overcome the limitation of peptide-based vaccination which requires functional immune system in the patients. In considering peptide vaccination, peptide dosage, site of injection and the selection of a suitable adjuvant are all critical factors that need to be determined in clinical trials. Alternatively, utilization of peptide-specific CTL clones could be utilized as a maintenance therapy post-autologous transplantation. A concern a self-antigen in the development of an XBP1 antigen-based immunotherapy is the potential for targeting normal plasma cells which express XBP1.<sup>30, 48</sup> However, the higher expression of XBP1 may make MM cells more specific target to the CTLs, but this issue should be closely monitored and investigated further in clinical trials.

In summary, we report here on heteroclitic XBP1<sub>184–192</sub> (YISPWILAV) and XBP1 SP<sub>367–375</sub> (YLFPQLISV) peptides with immunotherapeutic potential to efficiently target and lyse MM cells. These data provides information for their clinical application as either a peptide-based vaccination or adoptive transfer of *ex vivo* generated XBP1-specific T cells in the patients with MM and the pre-malignant diseases.

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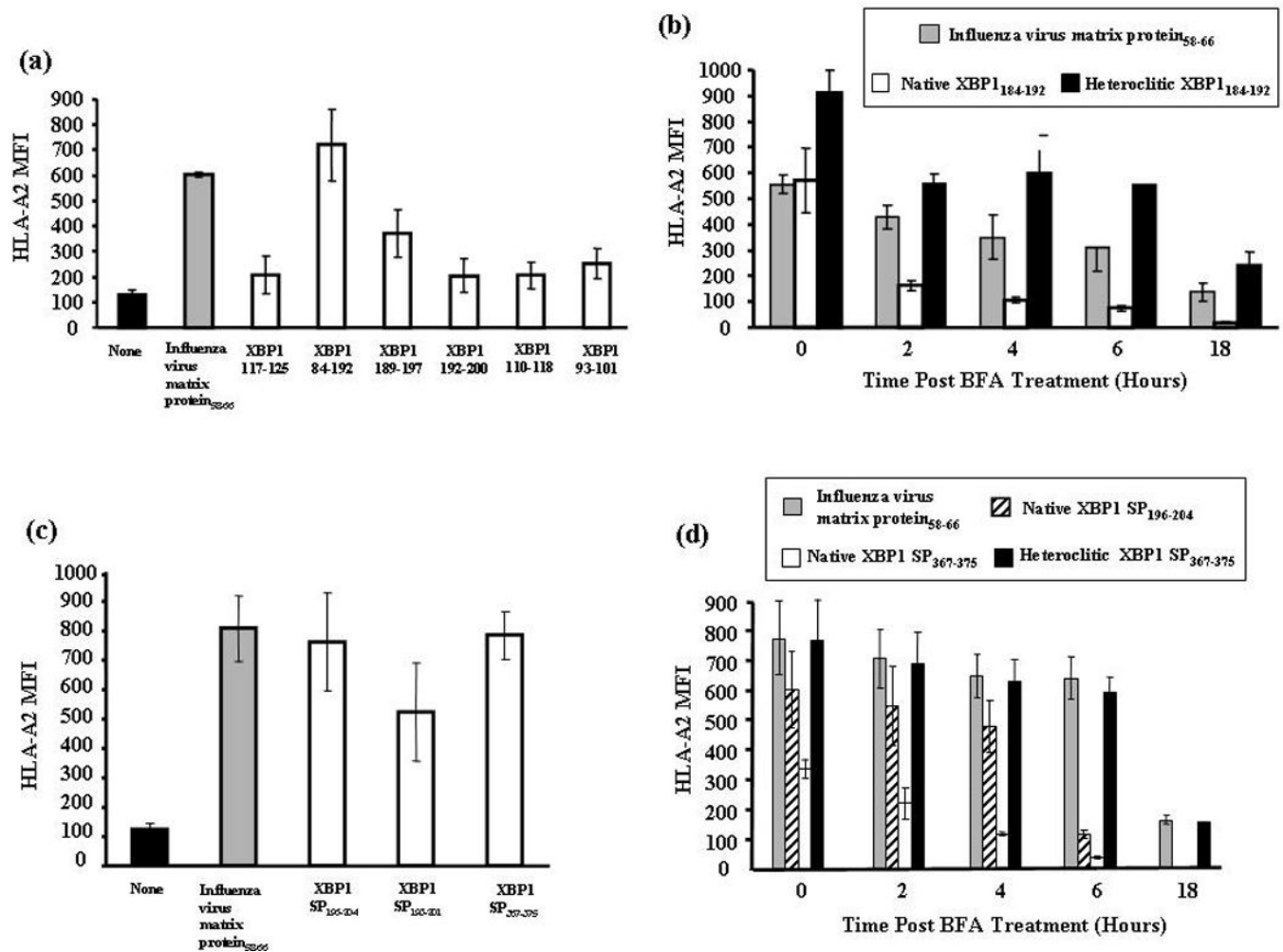
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**Figure 1. Identification of HLA-A2-specific XBP1 peptides and improvement of MHC binding stability by peptide modification**

Figure 1a. HLA-A2 binding affinity of non-spliced XBP1 peptides.

T2 cells were pulsed overnight with respective XBP1 peptide (50  $\mu$ g/ml) in serum-free media. Influenza virus matrix protein<sub>58-66</sub> was used as the positive control and T2 cell in media alone as baseline controls in these experiments. Following incubation, T2 cells were harvested, washed, and stained with HLA-A2-FITC mAb for flow cytometric analysis. HLA-A2 binding is shown as an increase in HLA-A2 mean fluorescence intensity (MFI). XBP1<sub>184-192</sub> (NISPWILAV) showed the highest HLA-A2 binding affinity among the non-spliced XBP1 peptides. The values represent the mean  $\pm$  SE of three separate experiments.

Figure 1b. HLA-A2 binding stability of non-spliced XBP1 peptides.

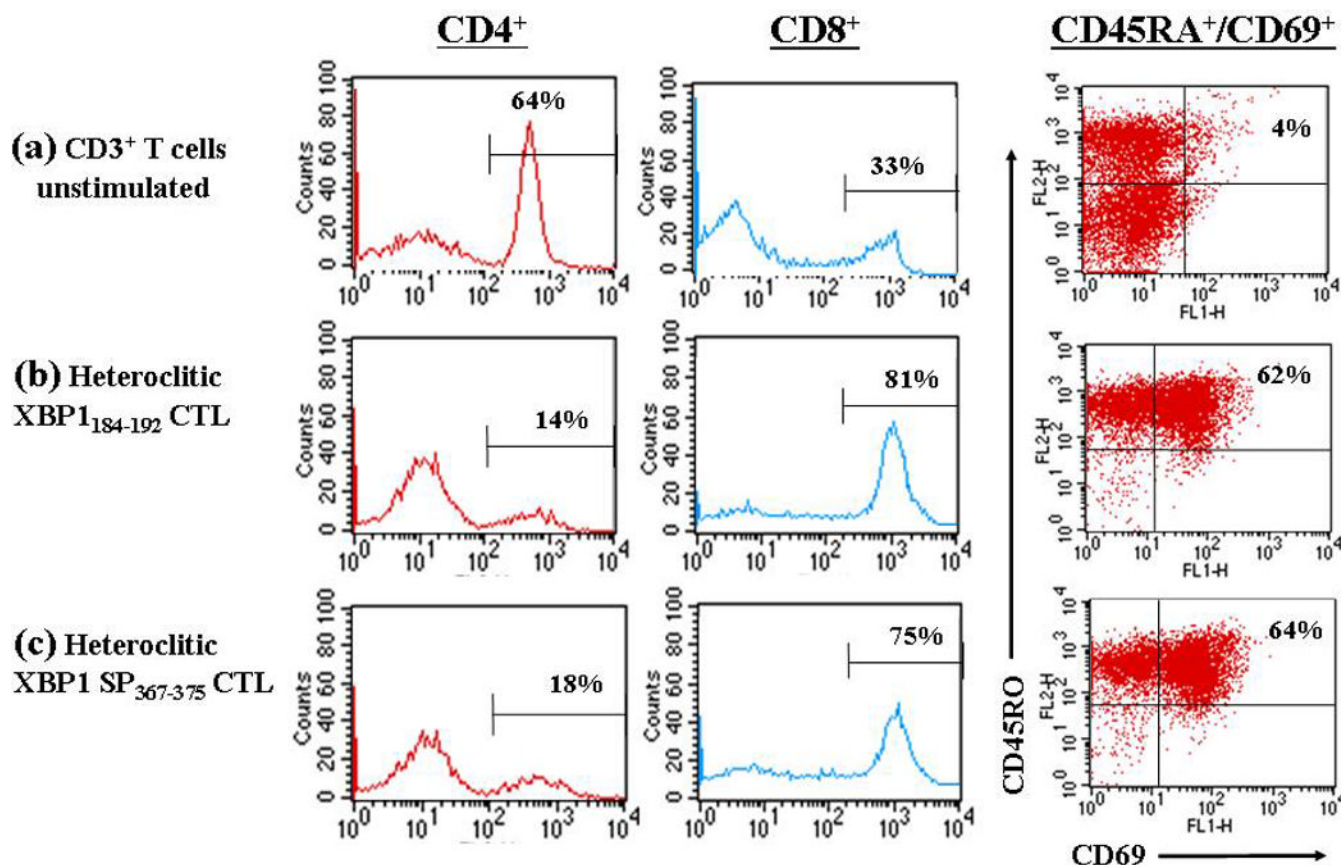
Native or heteroclitic XBP1 peptide (50  $\mu$ g/ml)-pulsed T2 cells were washed and incubated with Brefeldin A. At 0, 2, 4, 6, and 18 hours incubation, the cells were stained with HLA-A2-FITC mAb for flow cytometric analysis. Heteroclitic XBP1<sub>184-192</sub> (YISPWILAV) peptide showed increased HLA-A2 binding stability compared to the native XBP1<sub>184-192</sub> (NISPWILAV) peptide. Binding stability of the heteroclitic peptide was higher than influenza virus matrix protein<sub>58-66</sub> (GILGFVFTL), which was used as the HLA-A2-specific positive control peptide. The values represent the mean  $\pm$  SE of three separate experiments.

Figure 1c. HLA-A2 binding affinity of spliced XBP1 peptides.

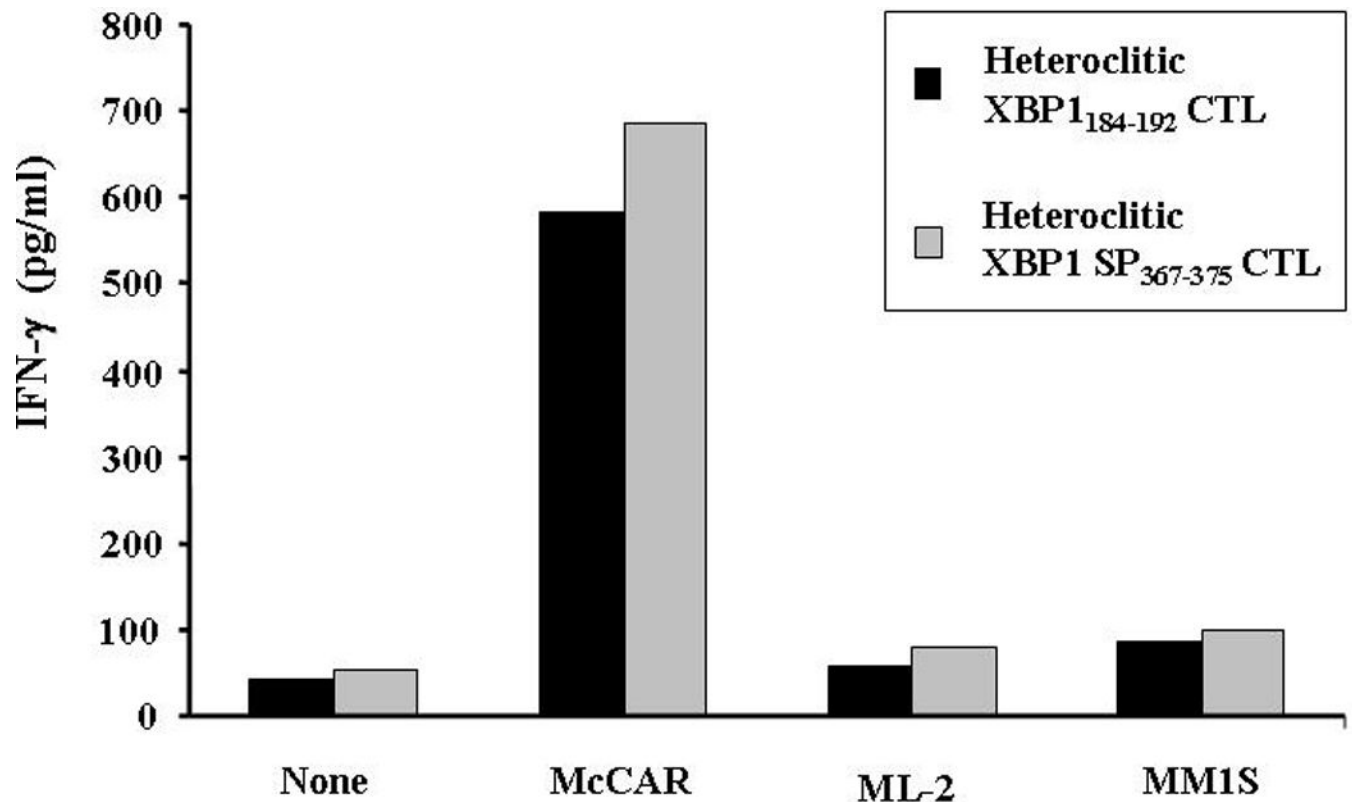
Spliced XBP1 peptides were evaluated for their HLA-A2 binding affinity as described in Figure 1a. XBP1 SP<sub>196–204</sub> (GILDNLDPV) and XBP1 SP<sub>367–375</sub> (ELFPQLISV) showed the highest HLA-A2 binding affinity among the spliced XBP1 peptides. The values represent the mean  $\pm$  SE of three separate experiments.

Figure 1d. HLA-A2 binding stability of spliced XBP1 peptides.

Spliced XBP1 peptides were analyzed for their HLA-A2 binding stability as discussed in Figure 1b. Heteroclitic XBP1 SP<sub>367–375</sub> (YLFPQLISV) peptide displayed increased HLA-A2 binding stability compared to its native XBP1 SP<sub>367–375</sub> (ELFPQLISV) or another native XBP1 SP<sub>196–204</sub> (GILDNLDPV) peptide. The values represent the mean  $\pm$  SE of three separate experiments.



**Figure 2. Distinct phenotype of CTLs stimulated with heteroclitic XBP1-specific peptides**  
 Enriched CD3<sup>+</sup> T cells obtained from a normal HLA-A2<sup>+</sup> donor were stimulated weekly with irradiated antigen-presenting cells pulsed with respective heteroclitic XBP1 peptide. One week after the 4<sup>th</sup> cycle of peptide stimulation, the CD3<sup>+</sup> T cells were analyzed for effector cells by flow cytometry. The percentage of CD4<sup>+</sup> helper T cells was decreased in the T cells stimulated with either (b) heteroclitic XBP1<sub>184-192</sub> **Y**ISPWILAV) peptide or (c) heteroclitic XBP1 SP<sub>367-375</sub> (**Y**LFPQLISV) peptide, compared to (a) no peptide. In contrast, the percentage of CD8<sup>+</sup> CTLs and CD69<sup>+</sup>/CD45RO<sup>+</sup> (activated memory) effector cells was increased in the CD3<sup>+</sup> T cells stimulated with respective XBP1 peptide, compared to no peptide control. The results are representative of three independent experiments.

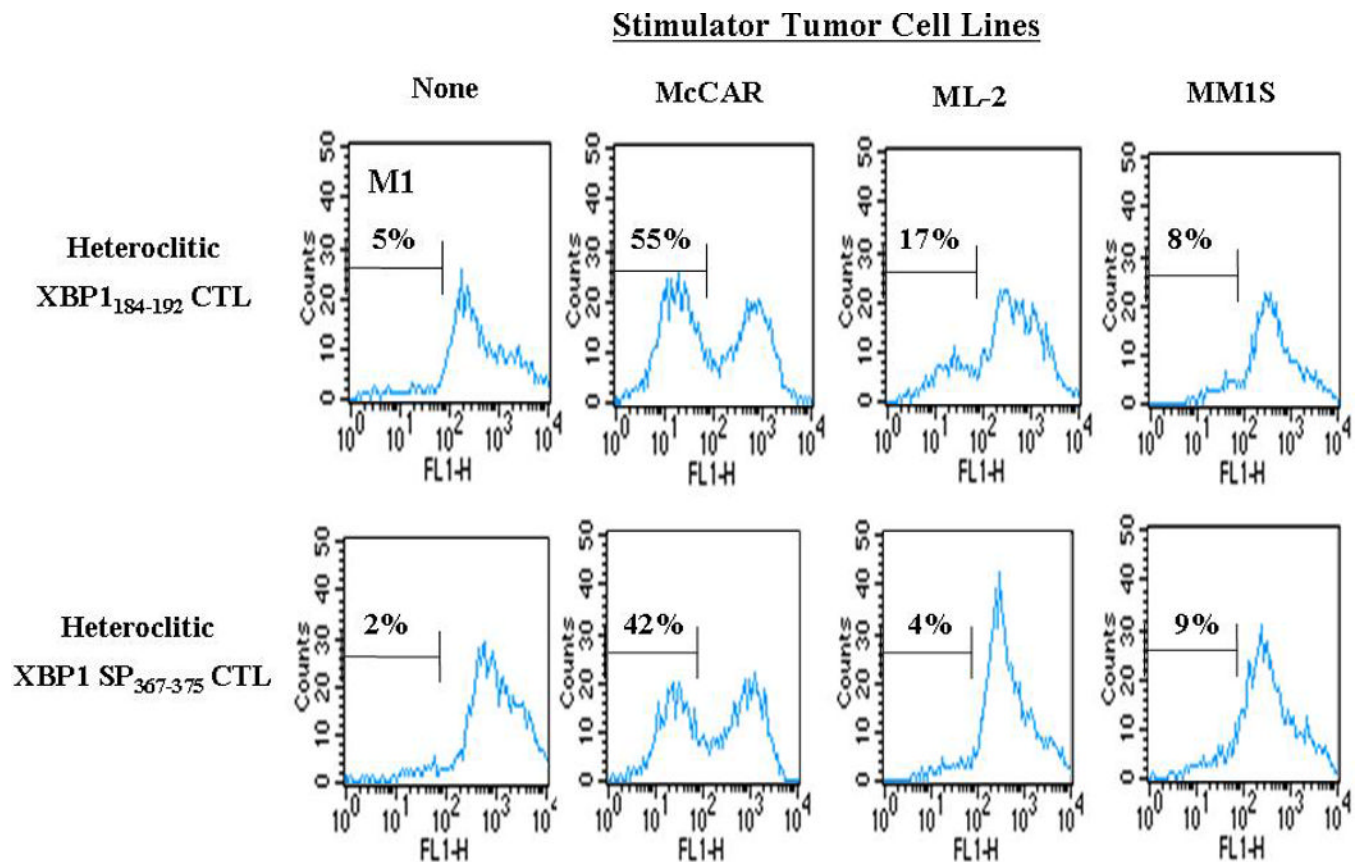


### Stimulator Tumor Cell Lines

**Figure 3. HLA-A2 restricted and XBP1-specific IFN- $\gamma$  secretion by XBP1-CTLs stimulated with heteroclitic peptides**

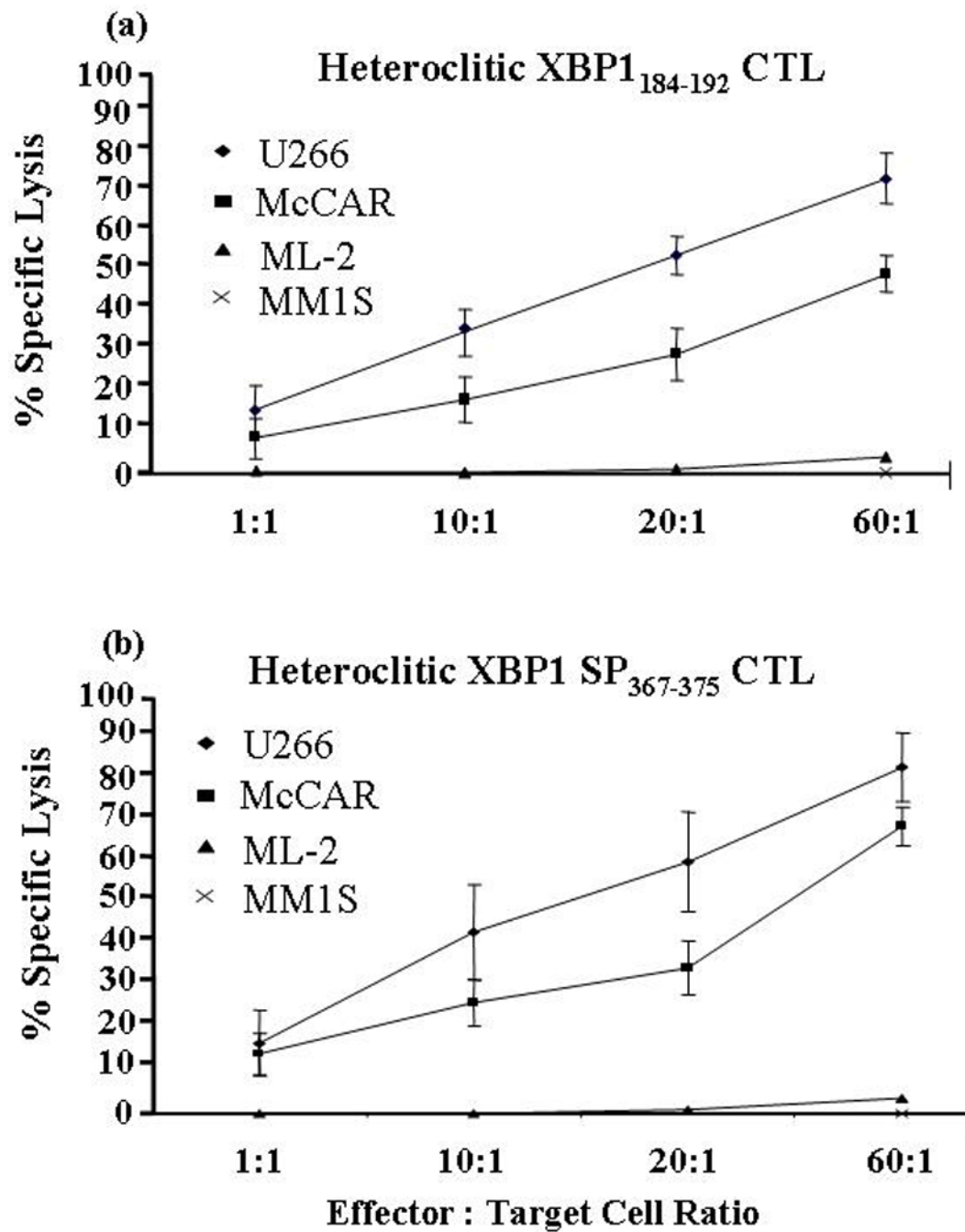
CTLs generated by repeated stimulation (4 $\times$ ) with heteroclitic XBP1<sub>184-192</sub> (YISPWILAV) or heteroclitic XBP1 SP<sub>367-375</sub> (YLFPQLISV) peptide were incubated with McCAR (MM; HLA-A2<sup>+</sup>/XBP1<sup>+</sup>), ML-2 (AML; HLA-A2<sup>+</sup>/XBP1<sup>-</sup>) or MM1S (MM; HLA-A2<sup>-</sup>/XBP1<sup>+</sup>) tumor cell lines. After 24 hours of incubation, the supernates were collected and IFN- $\gamma$  secretion was measured by ELISA. An increase in IFN- $\gamma$  secretion was detected in both XBP1-CTLs cultures in response to McCAR (HLA-A2<sup>+</sup>/XBP1<sup>+</sup>), but not to ML-2 (HLA-A2<sup>+</sup>/XBP1<sup>-</sup>) or MM1S (HLA-A2<sup>-</sup>/XBP1<sup>+</sup>) cells. XBP1-CTLs alone were used to examine the background IFN- $\gamma$  release. The results are representative of three independent experiments.





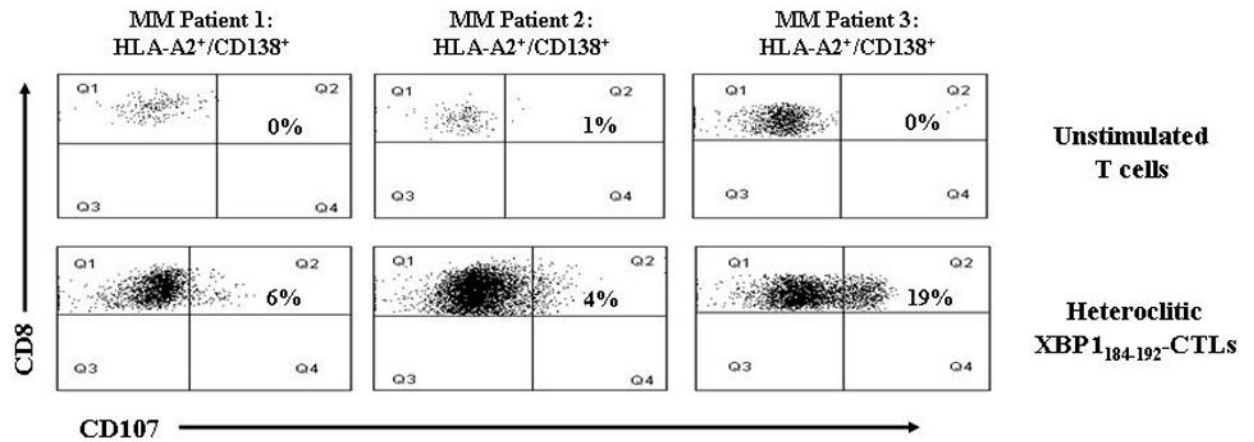
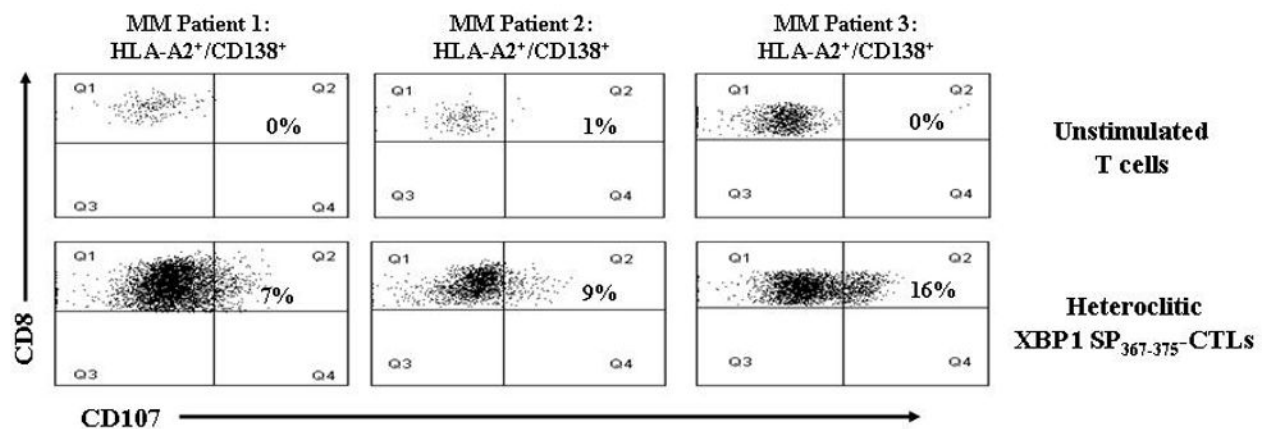
**Figure 4. Induction of HLA-A2-restricted and XBP1-specific cell proliferation of XBP1-CTLs in response to multiple myeloma cells**

Proliferation of CFSE-labeled XBP1-CTLs was measured in response to antigen stimulation by flow cytometry on day 4 of culture. The cell proliferation of heteroclitic XBP1<sub>184-192</sub> (YISPWILAV) and heteroclitic XBP1 SP<sub>367-375</sub> (YLFPQLISV) CTLs were both XBP1 antigen-specific and HLA-A2-restricted in response to the McCAR (HLA-A2<sup>+</sup>/XBP1<sup>+</sup>) myeloma cell line. The CTLs did not proliferate in response to either the XBP1 antigen or MHC mismatched cell lines ML-2 (HLA-A2<sup>+</sup>/XBP1<sup>-</sup>) or MM1S (HLA-A2<sup>-</sup>/XBP1<sup>+</sup>), respectively. Background proliferation was determined using CFSE labeled heteroclitic XBP1-CTLs cultured in media alone. The results are representative of three independent experiments.



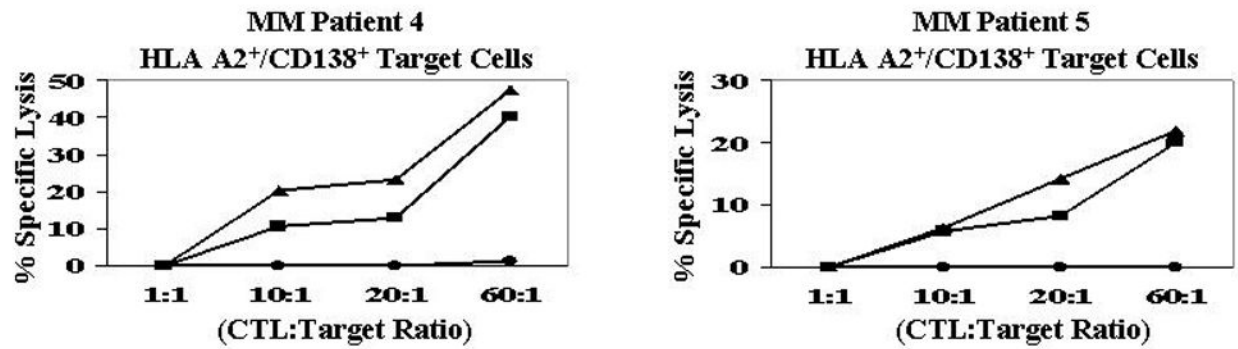
**Figure 5. HLA-A2-restricted and antigen-specific cytotoxicity of the XBP1-CTLs**

The specific cytotoxic activities of the heteroclitic XBP1-CTLs were analyzed one week after their 4<sup>th</sup> stimulation using the calcein cytotoxicity assay. A high level of cytotoxic activity against U266 (MM, HLA-A2<sup>+</sup>/XBP1<sup>+</sup>) (◆) and McCAR (MM, HLA-A2<sup>+</sup>/XBP1<sup>+</sup>) (■), but not against the ML-2 (AML, HLA-A2<sup>+</sup>/XBP1<sup>-</sup>) (▲) and MM1S (HLA-A2<sup>-</sup>/XBP1<sup>+</sup>) (×) cell lines was demonstrated by both the (a) heteroclitic XBP1<sub>184-192</sub> CTLs and (b) heteroclitic XBP1 SP<sub>367-375</sub> -CTLs. The values represent the mean±SE of three separate experiments.

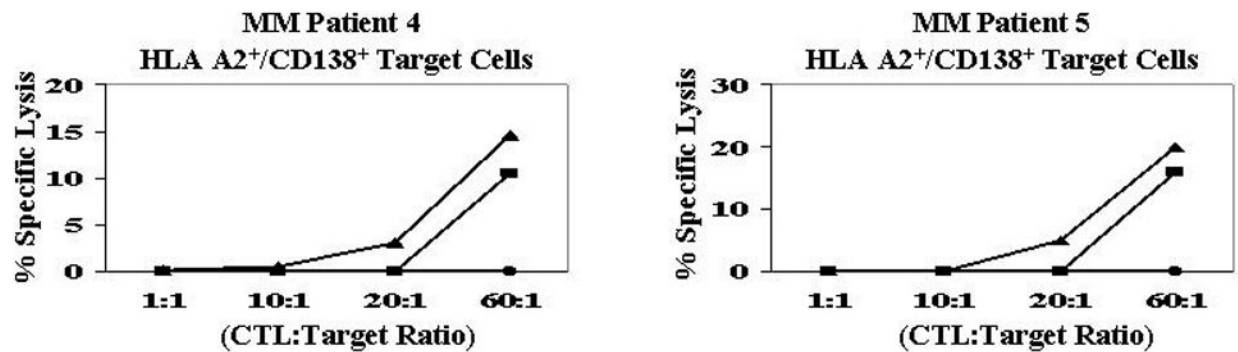
**a.****b.**

**Figure 6. XBP1-CTL degranulation in response to primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> patient MM cells**  
 Degranulation of heteroclitic XBP1-CTLs was analyzed by flow cytometry one week after their 4<sup>th</sup> stimulation, using CD107a, a surface antigen transiently present on the cell surface after release of cytotoxic granules. CD107a expression on (a) heteroclitic XBP1<sub>184-192</sub>-CTLs and (b) heteroclitic SP<sub>367-375</sub>-CTLs following stimulation with primary CD138<sup>+</sup> MM cells from three HLA-A2<sup>+</sup> patients. Control unstimulated T cells did not degranulate when exposed to the primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> MM cells. The results are expressed as the percent CD107a of CD8<sup>+</sup> T cells.

### a. Donor A CTLs



### b. Donor B CTLs



**Figure 7. Cytotoxicity of XBP1-CTLs against primary HLA-A2<sup>+</sup>/CD138<sup>+</sup> patient MM cells**  
The specific cytotoxic activity of heteroclitic XBP1-CTLs were analyzed one week after their 4<sup>th</sup> stimulation using the calcein-release cytotoxicity assay. Heteroclitic XBP1<sub>184-192</sub> CTLs (■) and heteroclitic SP<sub>367-375</sub>-CTLs (▲) generated from T cells of (a) Donor 1 and (b) Donor 2 demonstrate a high level of cytotoxic activity against primary CD138<sup>+</sup> MM cells from two HLA-A2<sup>+</sup> MM patients. Control unstimulated T cells (●) did not lyse primary CD138<sup>+</sup> MM cells from the HLA-A2<sup>+</sup> MM patients.

**Table 1**

Potential HLA-A2 Peptides Identified from Non-spliced XBP1 protein

PEPTIDES	SEQUENCE	SYFPEITHI	BIMAS	NetCTL
Non-spliced XBP1 <sub>117-125</sub>	LLREKTHGL	29	11.316	0.9257
Non-spliced XBP1 <sub>184-192</sub>	NISPWILAV	26	21.996	1.1996
Non-spliced XBP1 <sub>189-197</sub>	ILAVLTLQI	26	17.736	1.0263
Non-spliced XBP1 <sub>192-200</sub>	VLTLLQIQL	26	83.527	0.8323
Non-spliced XBP1 <sub>110-118</sub>	KLLLENQLL	23	276.643	1.001
Non-spliced XBP1 <sub>193-101</sub>	RMSELEQQV	21	205.951	0.9445
Non-spliced XBP1 <sub>200-208</sub>	LISCWAFWT	14	55.567	0.5846
Non-spliced XBP1 <sub>17-25</sub>	VLLLSGQPA	17	31.249	0.6260
Non-spliced XBP1 <sub>124-132</sub>	GLVVENQEL	23	21.362	0.7905
Non-spliced XBP1 <sub>19-27</sub>	LISGQPASA	22	8.446	0.7587



**Table 2**

Potential HLA-A2 Peptides Identified from Spliced XBP1 protein

PEPTIDES	SEQUENCE	SYFPEITHI	BIMAS	NetCTL
Spliced XBP1 <sub>196-204</sub>	GILDNLDPV	26	163.502	1.2062
Spliced XBP1 <sub>193-201</sub>	ILLGILDNL	30	151.434	1.1289
Spliced XBP1 <sub>367-145</sub>	ELFPQLISV	26	44.392	1.0892
Spliced XBP1 <sub>110-118</sub>	KLLLENQLL	23	276.643	1.001
Spliced XBP1 <sub>93-101</sub>	RMSELEQQV	21	205.951	0.9445
Spliced XBP1 <sub>189-197</sub>	SESDILLGI	17	1.913	0.3759
Spliced XBP1 <sub>238-246</sub>	SVGTSSAKL	20	1.869	0.4557
Spliced XBP1 <sub>330-338</sub>	CLLDAYSDC	13	111.047	0.2422
Spliced XBP1 <sub>263-271</sub>	LVLEIPSET	16	16.816	0.5069
Spliced XBP1 <sub>215-223</sub>	ASLEELPEV	23	15.841	0.9009